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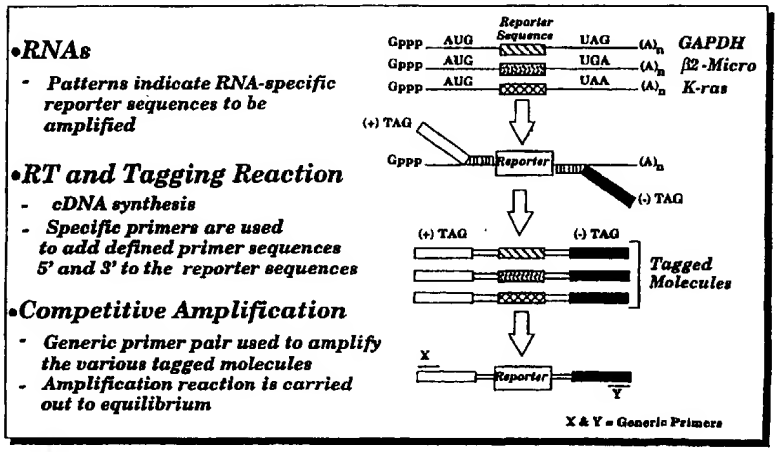
(54) Title: IMPROVED PROCESS FOR DETECTION AND QUANTIFICATION OF NUCLEIC ACID MOLECULES

Multiplex Competitive PCR Scheme



(57) Abstract

A process for measuring the relative amounts of two or more different nucleic acid molecules in a biological system using a multiplex competitive polymerase chain reaction.

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DESCRIPTION

IMPROVED PROCESS FOR DETECTION AND QUANTIFICATION OF NUCLEIC ACID MOLECULES

Background of the Invention

This invention relates to improved methods for detecting and quantifying desired nucleic acid molecules from a complex mixture of nucleic acids.

The following is a discussion of various methods for nucleic acid detection and quantitation. The discussion is not meant to be complete and is provided only for understanding of the invention that follows. This summary is not an admission that any of the work described below is prior art to the claimed invention.

Transcription of genes into messenger RNAs is the first step in the process of gene expression and represents a major site of gene regulation. Indeed, mRNA abundance is the most widely used parameter for quantifying gene expression. The two techniques most widely used to assay mRNA production in cells or tissues are Northern analysis (Maniatis *et al*, 1982, *Molecular Cloning--A Laboratory Manual*, Cold Spring Harbor Press) and ribonuclease protection assays (RPAs) (Berk *et al*, *1977*, Cell 12, 721; Myers *et al*, *1985*, *Science* 230, 1242). In both techniques, dynamic changes in mRNA expression from a particular gene are generally measured relative to other mRNAs derived from "house-keeping" genes. House-keeping genes are genes that are expressed in a wide variety of cell types, whose levels of expression do not change appreciably in response to external stimuli and/or whose levels of expression are comparable between different cell types. Coanalysis of

experimental or desired mRNAs with mRNAs from house-keeping genes serves to control for potential variability in sample RNA integrity and potential loading differences between different samples. Examples of house-keeping genes typically used as controls in Northern and RPA analyses are structural genes such as β-actin, major histocompatibility genes such as β2 microglobulin and genes encoding enzymes that are involved in important metabolic pathways such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the glycolysis pathway.

One of the limitations of the Northern and RPA techniques is their relatively poor sensitivity. RNAs expressed at less than 100 copies per cell are difficult to detect in total cellular RNA with either of these techniques. This is because mRNAs comprise less than 5% of the RNA found in most cell types. Thus, in order to analyze low abundance mRNAs, relatively large amounts of total RNA must be obtained and poly(A)+mRNAs must be purified from the total RNA in order to obtain sufficient amounts of the target RNA to analyze by Northern or RPA. The amounts of RNA required (usually over 100 micrograms) can be prohibitive when cell or tissue types being studied are rare, precious or scarce.

The problem of analyzing low abundance mRNAs was partially solved with the development of the quantitative polymerase chain reaction (PCR) techniques (Mullis, April 1990, *Sci. Am.*, 56; Arnheim *et al, 1992, Ann. Rev. Biochem.*, 61, 131). Two quantitative PCR methodologies have been developed - quantitative competitive PCR (QC-PCR) (Becker-Andre *et al.*, 1989, *Nucleic Acids Res.*, 17, 9437; Thompson *et al., 1992,*

Blood, 79, 1629; Beaudry *et al*, *1997*, *Meth. Mol. Biol.*, 74, ch:34) and kinetic PCR (Higuchi et *al*, *1992*, *Biotechnology*, *10*, 413; Higuchi *et al*, *1993*, *Biotechnology*, 11, 1026). In QC-PCR, the copy number of an RNA sequence in a given sample is quantified by titrating known amounts of a synthetic competitor into the sample RNA. The synthetic competitor is identical to the target sequence except for minor mutations used to distinguish its amplification product from that of the intended target mRNA sequence. Amplification product from the competitor is sufficiently similar to the product from the intended target to allow the two products to hybridize to form heterodimers. Thus, when product from either the target or the competitor accumulates to the point where they hybridize with each other faster than DNA polymerization can be initiated during the primer annealing step, then amplification of both the competitor and the intended target sequences ceases and the ratio of the two products becomes fixed. The amount of input competitor required to inhibit amplification of the intended target by 50% is generally used to determine the amount of target mRNA originally present in the sample. Drawbacks of the QC-PCR technique are that it is not readily adaptable to co-analysis of an internal house-keeping gene to control for differences in loading and integrity of the sample RNA and it is labor intensive since up to ten PCR reactions are usually required to generate a titration curve covering a broad enough range of input competitor to accurately quantify target mRNAs.

In the kinetic PCR technique, the rate of accumulation of product from the intended target RNA is

compared to the rate of accumulation of product from a control mRNA that is amplified from the same sample RNA but in a separate amplification reaction. Thus, unlike with the QC-PCR technique described above, differences in RNA loading and integrity can be controlled. However, manual quantification of product accumulation throughout the course of PCR amplification process (≥30 samples/reaction) can be labor intensive. A device that automatically monitors product accumulation has been introduced to the market recently (Gibson *et al., 1996, Genome Res., 6,* 995; Chiang *et al, 1996, Genome Res.* 6,1013) but the instrument and reagents are rather expensive.

A useful development in the field of RNA detection technology is the chip arrays by Affymetrix (Fodor *et al, 1993, Nature,* 364, 555; Lipshutz *et al., 1995, BioTechniques,* 19, 442). With this technology, thousands of different DNA oligonucleotides of known sequences can be synthesized in ordered arrays on small (<2cm) glass chips. These oligonucleotides can serve as capture probes to which cDNA probes generated from a particular tissue or cell type are hybridized. Thus, mRNA expression levels of thousands of different genes can be monitored in a single assay. While the chip technology is powerful for assaying many genes expressed within a single RNA sample, this assay would not likely be adequate as an endpoint for screening a large number of compounds for inhibitors of transcription of a given gene. This circumstance would require an assay capable of quantifying expression of one gene in a large number of samples.

Thus there exists in the field a need for an assay that is high throughput, that utilizes internal house-keeping gene(s) as controls for RNA input and integrity, and that has sufficient sensitivity to detect low-abundance mRNAs in small samples of RNA. The invention described in this application is intended to meet these needs.

The references cited above are distinct from the presently claimed invention since they do not disclose and/or contemplate the methods claimed and disclosed in the instant invention. Furthermore, Applicant believes that these references do not disclose the quantification and detection processes disclosed in this application.

Summary of the Invention

This invention concerns detection and quantification of any nucleic acid sequence of interest (desired nucleic acid or target nucleic acid) from a complex pool of nucleic acid sequences. Specifically, the invention relates to a process for rapid simultaneous amplification of two or more RNA molecules in a large number of biological samples. More specifically, the invention relates to a process for rapid quantification of expression of a desired gene by quantifying the level of RNA in a large number of biological samples. The methods disclosed in this invention also involve simultaneous amplification of a desired RNA sequence and one or more control RNA sequences, such as the house-keeping genes, in a single reaction mixture.

The invention concerns a process for measuring the relative amounts of two or more different nucleic acid

molecules in a system. The method includes: a) converting the nucleic acid molecules to tagged molecules using a 5'- and a 3'- primer specific for each nucleic acid molecule. The 5' primers each have a first defined sequence, and the 3' primers each have a second defined sequence; b) amplifying the tagged molecules together in one reaction vessel with a generic primer pair (where one generic primer has a sequence complementary to the first defined sequence, and the other generic primer has a sequence complementary to the second defined sequence), to produce amplified tagged nucleic acid molecules; and c) measuring the relative amount of each tagged molecule as a measure of the relative amount of different nucleic molecules.

In a preferred embodiment, the invention features a process for measuring the relative amounts of two or more different RNA molecules in a system.

In another preferred embodiment, the invention features a process for measuring the relative amounts of two or more different DNA molecules in a system.

For simplicity and ease of understanding the invention, Applicant refers to the invention scheme outlined in Figure 1A as "Multiplex Competitive PCR" or "MC-PCR." This technique provides a means to co-amplify, in a competitive manner and in a single reaction mixture, nucleic acid molecules, for example RNA, from a single biological sample, a desired RNA sequence and RNA sequences from one or more control genes, such as the house-keeping genes mentioned above.

The MC-PCR strategy combines: i) the utility of using internal RNAs as controls for RNA loading and integrity that is key to Northern, RPAs and kinetic PCR

strategies; ii) the precision of QC-PCR; and iii) the sensitivity of PCR amplification; all in a single reaction mixture without the need for the constant monitoring of amplification product accumulation that hampers other techniques, such as the kinetic PCR strategy.

In one preferred embodiment, the invention features a process for amplifying two or more RNA sequence by: a) cDNA synthesis (RT reaction); b) tagging reaction; and c) competitive amplification.

By "cDNA synthesis" or the Reverse Transcription (RT) reaction as used herein, is meant, conversion of a single stranded RNA sequence into its complementary DNA (cDNA) sequence (Figure 1A). The desired RNA sequence and control RNA sequences are converted into cDNA using enzymes, such as the reverse transcriptase. The RT step is carried out using DNA oligonucleotide primers specific for each target RNA sequence. This process introduces sequences downstream from the primers, which are suitable substrates for subsequent PCR amplification.

By "tagging" reaction (Figure 1A) as used herein, is meant introducing known sequence tags at the ends of the sequence to be amplified (Reporter sequence). The Tagging reaction serves two purposes. The first purpose is to generate a second strand of DNA using the cDNA generated in the RT step as the template. The second purpose of the tagging reaction is to attach defined sequences at the 5'- and 3'-ends of the molecule (first defined sequence and second defined sequence, respectively). These defined sequences, also referred to as the 5'-TAG sequences (first defined sequence) and

3'-TAG sequences (second defined sequence), are added, during the tagging reaction, to each of the reporter sequences being amplified to generate "tagged molecules". Two DNA oligonucleotides - termed the "plus strand TAG primers" (5'-primer) and "minus strand TAG primers" (3'-primer) specific for each target RNA are used to accomplish this. The TAG sequence included in the 5'-primers is referred to as the (+)**TAG** (first defined sequence), while the downstream primers (3'-primer) are referred to as (-)**TAG** second defined sequencer (Figure 1A).

In non-limiting examples provided in this application, the TAG sequences used are about 60 nt in length. Generally it is preferred that such sequences have a length between 17 and 150 bases, preferably about 40-70 bases. The specific length of the TAG sequences disclosed in the example are not limiting in the invention and those skilled in the art will recognize that the length and sequence the TAG sequences can be significantly varied without significantly effecting the invention. The sequences between the plus strand and negative strand TAG primers are referred to as the "reporter sequences." These reporter sequences are unique to each RNA being amplified. In the nonlimiting proof of principle studies described below, 24-nt regions were amplified from each mRNA to serve as the reporter sequences. The specific length of the reporter sequences disclosed in the example are not limiting in the invention and those skilled in the art will recognize that the length and sequence the reporter sequences can be significantly varied without significantly effecting the invention. Generally is it

preferred that such sequences have a length between 17 and 150 bases, preferably about 40-70 bases.

The DNA products, such as those disclosed in the non-limiting examples herein, generated after the tagging step are 180-bp long. These DNA products all contain the same **(+)TAG** sequence (60-bp long), followed by a 60 bp region specific for each target RNA (of which 24 bp corresponds to the reporter sequences specifically amplified by the TAG primers), followed by the same **(-)TAG** (60 bp; Figure 1C). In the examples provided below, a single target RNA is co-amplified with two control house-keeping RNAs producing three different products. These three different products are identical in their 5' and 3' ends but differ in their internal regions. Those skilled in the art will recognize that the length of the DNA products generated after the tagging reaction, the length of the reporter sequence, the length of the TAG sequence and the length of the sequence flanking the reporter sequence can all be varied without significantly effecting the disclosed invention and hence are covered by the invention.

By "competitive amplification" reaction is meant, a reaction where a single pair of primers, termed the "amplification primers" or "generic primers", specific for the **(+)TAG** and **(-)TAG** sequences (where one generic primer has a sequence complementary to the first defined sequence {**(+)TAG**} and the second generic primer has a sequence complementary to the second defined sequence {**(-)TAG**} are used to amplify the different products generated from target RNAs during the tagging step (tagged molecules). This amplification reaction is competitive due to the use of a single primer pair to

amplify the different target RNAS. In addition, the presence of the common **(+)TAG** and **(-)TAG** sequences on the ends of different products promotes the formation of heterodimers among the various products (Figure 1 B).

As described above, when a single sequence is amplified by PCR, reaction product accumulates to on an average 0.2 μM; at this point the rate of primer annealing during the annealing step is approximately the same as the rate of re-hybridization of the two complementary product strands. Consequently there is a limit to the amount of product generated in a PCR amplification reaction - exponential amplification ceases at or below 0.2 μM regardless of how much primer or other substrates are present in the reaction. If two or more different products are co-amplified, then each of the products will accumulate to about 0.2 μM and then cease to be amplified. Thus the total amount of product is greater in multiplex reactions (reactions where 2 or more; however, each product only accumulates to about 0.2 μM. This is not the case in MC-PCR because the products produced from the target and control RNAs contain a sufficient amount of common sequence -- due to the presence of the **(+)TAG** and **(-)TAG** sequences contained on all products in the reaction -- that heterodimer formation is possible (Figure 1B). Thus, once the sum of all three products reaches the critical concentration (approximately 0.2 μM), amplification of all three products ceases. No further amplification of the products occurs and the ratio of the different products is preserved. These ratios can be determined by quantifying the amount of reporter sequences present in the MC-PCR products. Differences in amounts of a

target RNAs in different samples can thus be determined by comparing the ratios of the products generated by the MC-PCR. While a wide variety of techniques can be used to accomplish this, in the examples provided below, MC-PCR products were immobilized on nitrocellulose filters and hybridized with probes specific for each reporter sequence.

The invention also features kits for use in the claimed methodology. Specifically, these kits include primers and probes and enzymes as well as buffers necessary for practice of the method. In preferred embodiments the kit includes a plurality of 5' and 3' primers as described above and a generic primer pair as described above. If desired, the appropriate components for measurement of the relative amount of amplified tagged molecules, may also be provided. The enzymes that might be included include reverse transcriptase, DNA polymerase as well as the substrates for such enzymes including dNTPs, rNTPs, if desired, and appropriate labeled molecules to allow measurement of relative amount of amplified taq molecules.

In more preferred embodiments, such kits may include 5' and 3' primers designed for amplification of household genes along with one or more 5' and 3' primers designed for amplification of the gene of interest. One or more generic primer pairs may also be provided in such a kit as noted above.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Drawings

The drawings will first briefly be described.

Drawings

Figures 1A-C. Schematic representation of MC-PCR Assay. **(A)** Steps involved in the MC-PCR assay. **(B)** Complexes that form between multiplexed PCR products during the competitive phase of MC-PCR assay. **(C)** Schematic representation of MC-PCR products generated in the following examples.

Figures 2A-D. Comparison of Northern, RPA and MC-PCR analysis of a time course of stromelysin mRNA induction in IL-1-treated HS27 human fibroblasts. (A-C) Northern, RPA and MC-PCR raw data are shown in Figs. 2A-C, respectively. 0, 7, 11, 15, 17 and 23, indicate hours post-induction with IL-1. (D.) Quantification of the magnitudes of stromelysin mRNA induction relative to untreated ("0"), as measured by these three different techniques.

Figures 3A-C. Sensitivity of MC-PCR assay. Stromelysin mRNA was induced to high levels in HS27 human fibroblasts by treatment with IL-1. Total RNA was extracted from the cells either prior to IL-1 treatment ("Uninduced" lanes) or 17 hours after IL-1 treatment ("Induced" lanes). **(A)** 5 µg of total HS27 RNA was analyzed by Northern blotting and hybridized with radio-labeled probes specific to stromelysin mRNA. Probes for 28S ribosomal and β2-microglobulin RNAs were included as controls for loading differences and RNA integrity. **(B)** Stromelysin mRNA induction was measured relative to β2-microglobulin and GAPDH in 1 µg of total HS27 RNA (in triplicate) by MC-PCR as described *infra*. **(C)** Varying

amounts of input HS27 total RNA, ranging from 10 ng to 1 µg, from either untreated ("Uninduced") and IL1-stimulated ("Induced") HS27 human fibroblasts (see parts **A** & **B**), was analyzed by MC-PCR for stromelysin mRNA induction. Stromelysin mRNA levels were analyzed relative to endogenous β2-microglobulin and GAPDH mRNAs.

Figure 4. MC-PCR analysis of admixtures of HS27 RNA from IL1-treated ("Induced") and untreated ("Uninduced") HS27 human fibroblasts. Total mRNA from IL1-stimulated HS27 cells (Figure 3) was diluted with total RNA from untreated cells (Figure 3) and analyzed by MC-PCR for Stromelysin mRNA levels.

Figures 5A-B. Comparison of Northern and MC-PCR analyses of serum induction of *c-fos* mRNA in serum-starved HeLa cells. (A.) Time-course of *c-fos* mRNA induction in serum-starved HeLa cells following serum stimulation as determined by Northern analysis. (B.) MC-PCR analysis of *c-fos* mRNA levels in serum-starved ("Unstimulated") HeLa cells and cells stimulated with serum for 30 min. Fos mRNA levels were analyzed relative to cdc2 kinase and *raf* kinase mRNAs as controls. Levels of cdc2 kinase and *raf* kinase mRNAs remain constant in HeLa cells during the time-frame of the experiment.

Figure 6. MC-PCR analysis of antisense inhibition of cdc2 kinase mRNA. HeLa cells were treated for 4 hours with either a phosphorothioate antisense oligonucleotide targeting cdc2 kinase mRNA or a mismatch control, complexed at a 4:1 charge ratio with Pfx4 (Life Technologies) in OptiMEM®. Cells were then washed, cultured for an additional 20 hours in complete media, RNA was harvested and 400 ng was analyzed by MC-PCR

using oligonucleotides specific for cdc2 kinase, and K-ras (ras) as a control target RNA. Error bars represent standard deviations.

Detailed Description of the Preferred Embodiments

By "amplification" as used herein, is meant, production of any particular sequence from a given sequence of RNA in amounts greater than the amount initially present.

By "co-amplification" is meant simultaneous amplification of more than one desired RNA sequence in a single reaction.

By "oligonucleotide" as used herein, is meant a molecule comprising two or more nucleotides preferably at least 11 nucleotides, most preferably at least 17 nucleotides.

By "primers" as used herein, is meant oligonucleotides comprising sequences that are complementary to a portion of the target sequence to be amplified, wherein said complementary sequence is capable of interacting with the target sequence by base-pairing interactions. This complementarity functions to allow sufficient hybridization of the primer molecule to the target sequence to allow the primer to be extended. Primer extension occurs when the primer and the target sequence are incubated under conditions suitable for primer extension in the presence of appropriate enzymes and nucleotides. One hundred percent complementarity is preferred, but complementarity as low as 50-75% may also be useful in this invention.

By "amplification primers" is meant primers comprising sequence that are complementary to either the

(+)TAG sequence (first defined sequence) or the **(-)TAG** sequence (second defined sequence). These primers are used during the competitive amplification reaction. These primers are also referred to as ligeneric primers".

By "complementary" is meant a nucleic acid sequence that can form hydrogen bond(s) with other nucleic acid sequence by either traditional Watson-Crick or other non-traditional types (for example, Hoogsteen type) of base-pairing interaction.

By "PCR" is meant polymerase chain reaction; PCR is method used to selectively amplify a desired nucleic acid sequence (for a review see Mullis, *1990, supra; Mullis et al.,* US Patent No. 4,683,195).

By "biological sample" (system) is meant material, in a purified or unpurified form, from biological sources, including but not limited to human, animal, plant, bacteria, viruses, fungi and the like, that contains the desired target RNA sequence(s) to be detected and/or amplified.

The instant invention involves detection and quantification of one or more desired RNA sequences from a complex pool of RNA sequences. Purified or unpurified RNA samples can be utilized as the starting material to practice the invention. The starting RNA sample may contain more than one desired target RNA sequence which may be the same or different.

The present invention is useful for generating large amounts of a desired RNA sequence and/or more than one desired RNA sequence, which may be same or different, located on the same or different RNA molecules.

Specifically, the invention relates to a process for rapid high-throughput amplification of a target RNAs in a large number of biological samples. More specifically, the invention relates to a process for rapid quantification of expression of a target gene(s) by quantifying the level of mRNA in a large number of biological samples.

In a preferred embodiment this invention involves simultaneous amplification of a desired RNA sequence and one or more control RNA sequences, such as the house-keeping genes, in a single reaction mixture.

Example 1: Comparison of Northern, RPA and MC-PCR techniques

Human fibroblasts normally express very low levels of the metalloprotease, stromelysin. However, fibroblasts produce large amounts of stromelysin mRNA and protein in response to IL-1 treatment. Stromelysin mRNA induction in HS27 human foreskin fibroblasts was used as a model system to demonstrate the MC-PCR assay.

Materials and methods:

Human foreskin fibroblasts cell line (HS-27) or primary human synovial fibroblasts (HSF) were used in this assay. All cells were plated the day before the assay in media containing 10% fetal bovine serum in 24 well plates at a density of $5x10^4$ cells/well. At 24 hours after plating, the media was removed from the wells and the monolayers were washed with Dulbeccos phosphate buffered saline (PBS). The cells were serum starved for 24 h by incubating the cells in media containing 0.5% fetal bovine serum (FBS; 1 ml/well).

Cells were washed twice with PBS containing Ca^{2+} and Mg^{2+}. Cells were then treated with FBS to a final concentration of 10%. Supernatants were harvested 16 hours after IL-1 induction and assayed for desired RNA expression by either RPA, Northern or MC-PCR.

Northern Analysis: Total RNA is extracted with RNeasy kits (Qiagen), and was analyzed by Northern-blot assay. Briefly, 0.5 µg cellular RNA was separated on 1.0 % agarose/formaldehyde gel and transferred to Zeta-Probe GT nylon membrane (Bio-Rad, Hercules, CA) by capillary transfer for ~16 hours. The blots were baked for two hours and then pre-hybridized for 2 hours at 65°C in 10 ml Church hybridization buffer (7 % SDS, 500 mM phosphate, 1 mM EDTA, 1 % Bovine Serum Albumin). The blots were hybridized at 65°C for ~16 hours with 10^6 cpm/mi of full length ^{32}P-labeled complementary RNA (cRNA) probes to stromelysin, β2-microglobulin, GAPDH and other RNAs (cRNA added to the pre-hybridization buffer along with 100 µl 10 mg/ml salmon sperm DNA). The blot was rinsed once with 5% SDS, 25 mM phosphate, 1 mM EDTA and 0.5% BSA for 10 min at room temperature. This was followed by two washes (10 min each wash) with the same buffer at 65°C, which was then followed by two washes (10 min each wash) at 65°C with 1% SDS, 25 mM phosphate and 1 mM EDTA. The blot was autoradiographed. The blot was reprobed with a 100 nt cRNA probe to 18S rRNA as described above. Following autoradiography, the stromelysin expression was quantified by PhosphorImaging, which is followed by normalization of the data to the 18S rRNA band intensities.

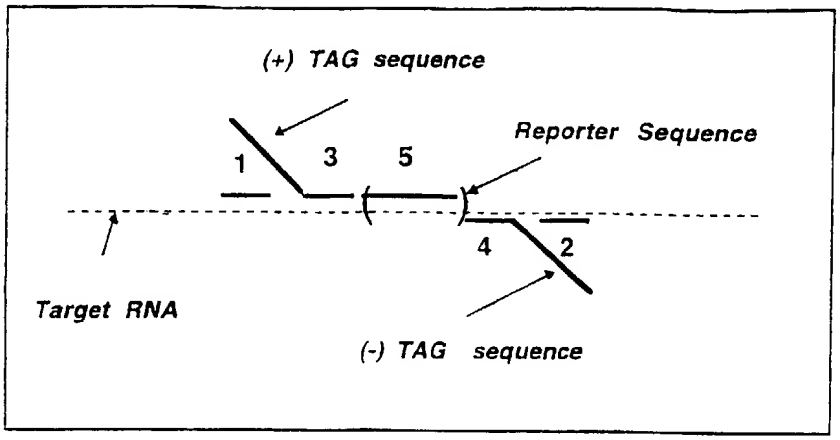
RNase protection analysis: The assay is carried out essentially as described in the protocol provided with

the Lysate Ribonuclease Protection Kit (United States Biochemical Corp.) The probe for RNase protection is an RNA that is complementary to the desired RNA sequence. This "antisense" probe RNA is transcribed *in vitro* from a template prepared by the polymerase chain reaction in which the 5' primer was a DNA oligonucleotide containing the T7 promoter sequence. The probe RNA is body labeled during transcription by including ^{32}P[CTP] in the reaction and purified away from unincorporated nucleotide triphosphates by chromatography on G-50 Sephadex. The probe RNA (100,000 to 250,000 cpms) is allowed to hybridize overnight at 37°C to the RNA from a cellular lysate or to RNA purified from a cell lysate. After hybridization, RNAse T_1 and RNAse A are added to degrade all single-stranded RNA and the resulting products are analyzed by gel electrophoresis and autoradiography. RNAse protection analysis was carried out on cellular RNA isolated from human synovial fibroblasts that had been treated with IL-1 or untreated.

MMC-PCR assay:

Oligonucleotides

Oligonucleotides labeled **#1** through **#5** indicated in the diagram below were used in the present studies to accomplish the MC-PCR.



Oligonucleotides labeled with a **#2** correspond to the primer used specifically for reverse transcription of a target mRNA. Oligonucleotides labeled with a **#1** are plus-strand primers used in combination with primer # **2** to convert the region of the target mRNA to be amplified during the MC-PCR reaction into double-stranded DNA. (Note that conversion of the CDNA into double-stranded DNA using primer **#1** is not indicated in Figure 1A and C. This step was performed in the present studies to increase specificity of amplification of the reporter sequences. We have found that this step is not required for high-abundance mRNAs but may be necessary for low-abundance mRNAs, ie., less than 100 copies/cell). Primers **#3** and **#4** are nested relative to primers **#1** and **#2** and represent the tagging primers referred to in Figure 1A. Oligonucleotides labeled **#5** are the probes used to detect the reporter sequences specifically amplified by primers #**3** and **#4**. The defined sequences included in the tagging primers **#3** and **#4** and used during the competitive amplification step (Figure 1A) are referred to as **(+)TAG primer** sequence (5' ACA TTC TCA AGT CTG ACT TTG CCT TTG CAT AAC AAG CAC TTA GCA TTA ACC CTC ACT AAA 3' SEQ. ID. NO.: 37) and the **(-)TAG** sequence (5'

ATT CGA ACT TCT CGA TTC GAA CTT CTG ATA GAC TTC GAA ATT AAT ACG ACT CAC TAT AGG 3' SEQ. ID. NO.: 38).

cdc2 oligos:

cdc-1 5'- CAG ACT AGA AAG TGA AGA-3'
(SEQ. ID. NO.: 1)
cdc-2 5'- CCA TGT ACT GAC CAG GAG-3'
(SEQ. ID. NO.: 2)
cdc-3 5'- ACA TTC TCA AGT CTG ACT TTG CCT TTG CAT AAC AAG CAC TTA GCA TTA ACC CTC ACT AAA CGT CAT CCA AAT ATA GTC-3'
(SEQ. ID. NO.: 3)
cdc-4 5'- ATT CGA ACT TCT CGA TTC GAA CTT CTG ATA GAC TTC GAA ATT AAT ACG ACT CAC TAT AGG GAG ATA TAA CCT GGA ATC-3'
(SEQ. ID. NO.: 4)
cdc-5 5'- AGT CTT CAG GAT GTG CTT ATG CAG-3'
(SEQ. ID. NO.: 5)

K-ras oligos:

ras-1 5'- TAC AGC TAA TTC AGA ATC-3'
(SEQ. ID. NO.: 6)
ras-2 5'- CAA GAG ACA GGT TTC TCC-3'
(SEQ. ID. NO.: 7)
ras-3 5'- ACA TTC TCA AGT CTG ACT TTG CCT TTG CAT AAC AAG CAC TTA GCA TTA ACC CTC ACT AAA ATT TTG TGG ACG AAT ATG-3'
(SEQ. ID. NO.: 8)
ras-4 5'- ATT CGA ACT TCT CGA TTC GAA CTT CTG ATA GAC TTC AA ATT AAT ACG ACT CAC TAT AGG TCA TCA ATT ACT ACT TGC TT-3'
(SEQ. ID. NO.: 9)

ras-5 5'- TCC AAC AAT AGA GGA TTC CTA CAG G-3'
(SEQ. ID. NO.: 10)

C-fos oligos:

fos-1 5'- TAA GAT GGC TGC AGC CGA-3'
(SEQ. ID. NO.: 11)
fos-2 5'- CAG TTT GGC AAT CTC TGT CTG-3'
(SEQ. ID. NO.: 12)
fos-3 5'- ACA TTC TCA AGT CTG ACT TTG CCT TTG CAT AAC AAG CAC TTA GCA TTA ACC CTC ACT AAA GGC GAA ATC GGA GGA GGG-3'
(SEQ. ID. NO.: 13)
fos-4 5'- ATT CGA ACT TCT CGA TTC GAA CTT CTG ATA GAC TTC GAA ATT AAT ACG ACT CAC TAT AGG CAA XGC AGA CTT CTC ATC TTC-3'
(SEQ. ID. NO.: 14)
fos-5 5'- ACA CTC CAA GCG GAG ACA GAT CAA-3'
(SEQ. ID. NO.: 15)

c-raf oligos:

raf-1 5'- TCG TAT GCG AGA GTC TGT-3'
(SEQ. ID. NO.: 16)
raf-2 5'- CCT TCA GAT GAG GGA CTG-3'
(SEQ. ID. NO.: 17)
raf-3 5'- ACA TTC TCA AGT CTG ACT TTG CCT TTG CAT AAC AAG CAC TTA GCA TTA ACC CTC ACT AAA CAC TTA GCA TTA ACC CTC ACT AAA TTC CAG GAT GCC TGT TAG T-3'
(SEQ. ID. NO.: 18)

raf-4 5'- ATT CGA ACT TCT CGA TTC GAA CTT CTG
ATA GAC TTC GAA ATT AAT ACG ACT CAC
TAT AGG GAG GTG TTA AAG GTG AAG-3'
(SEQ. ID. NO.: 19)
raf-5 5'- GCA CAG ATA TTC TAC ACC TCA CGC-3'
(SEQ. ID. NO.: 20)

β2-Microglobulin oligos:

B1 5'- TAG CTG TGC TCG CGC TAC
(SEQ. ID. NO.: 21)
B2 5'- TTT CCA TTC TCT GCT GGA
(SEQ. ID. NO.: 22)
B3 5'- ACA TTC TCA AGT CTG ACT TTG CCT TTG
CAT AAC AAG CAC TTA GCA TTA ACC CTC
ACT AAA TCT CTC TTT CTG GCC TGG-3'
(SEQ. ID. NO.: 23)
B4 5'- ATT CGA ACT TCT CGA TTC GAA CTT CTG
ATA GAC TTC GAA ATT AAT ACG ACT CAC
TAT AGG TGA CGT GAG TAA ACC TGA A-3'
(SEQ. ID. NO.: 24)
B5 5'- AGG GTA TCC AGC GTA CTC CAA AGA-3'
(SEQ. ID. NO.: 25)

GAPDH oligos:

G1 5'- GCC ACA TCG CTC AGA CAC-3'
(SEQ. ID. NO.: 26)
G2 5'- CCA GAG TTA AAA GCA GCC-3'
(SEQ. ID. NO.: 27)
G3 5'- ACA TTC TCA AGT CTG ACT TTG CCT TTG
CAT AAC AAG CAC TTA GCA TTA ACC CTC
ACT AAA CAT GGG GAA GGT GAA GGT-3'
(SEQ. ID. NO.: 28)

G4 5'- ATT CGA ACT TCT CGA TTC GAA CTT CTG ATA GAC TTC GAA ATT AAT ACG ACT CAC TAT AGG CTG GTG ACC AGG CGC CCA-3'

(SEQ. ID. NO.: 29)

G-5 5'- CGG AGT CAA CGG ATT TGG TCG TAT-3'

(SEQ. ID. NO.: 30)

Stromelysin oligos:

S1 5'- TGC TGC TCA TGA AAT TGG-3'

(SEQ. ID. NO.: 31)

S2 5'- CAG GCG GAA CCG AGT CAG-3'

(SEQ. ID. NO.: 32)

S3 5'- ACA TTC TCA AGT CTG ACT TTG CCT TTG CAT AAC AAG CAC TTA GCA TTA ACC CTC ACT AAA CCC TGG GTC TCT TTC ACT-3'

(SEQ. ID. NO.: 33)

S3 5'- ACA TTC TCA AGT CTG ACT TTG CCT TTG CAT AAC AAG CAC TTA GCA TTA ACC CTC ACT AAA CCA CTC CCT GGG TCT CTT-3'

(SEQ. ID. NO.: 34)

S4 5'- ATT CGA ACT TCT CGA TTC C-AA CTT CTG ATA GAC TTC GAA ATT -ZKAT ACG ACT CAC TAT AGG GTC TGT GAG TGA GTG ATA-3'

(SEQ. ID. NO.: 35)

S5 5'- CAG CCA ACA CTG AAG CTT TGA TGT-3'

(SEQ. ID. NO.: 36)

Sequences listed above and in the application are meant to be nonlimiting examples. Those skilled in the art will recognize that variants of these sequences can be readily generated using techniques known in the art, and are within the scope of the present invention.

MC-PCR Methodology

RNA purification. RNA was purified using the RNeasy kits (Qiagen).

Reverse transcription and conversion to double stranded DNA. Sample RNA, ranging from 10 ng to 1 µg in 5 µl of water, was combined in thin-walled 0.5ml Eppendorf microcentrifuge tubes with 1 µl of a RT primer mix consisting of 5 µM of primer #2 for each target to be analyzed. The resulting mixtures were heated to 90EC for 3 minutes, then slow-cooled on the bench top to promote hybridization of primers #2 to the target RNA. Reverse transcription was accomplished by adding 20 ml of RT Mix consisting of 1.36X PCR reaction buffer (Perkin Elmer), 10 U AMV reverse transcriptase (Boehringer Mannheim),0.2 mM dNTPs, 25 U RNase inhibitor (Boehringer Mannheim), 1 U of AmpliTaq Gold (Perkin Elmer) and 0.27 mM primer #1 for each target, and heating to 42EC for 1 h. The cDNAs were then converted into double-stranded DNA primed by oligonucleotide #1 included in the RT Mix by heating the reactions to 94°C for 10 min to activate the AmpliTaq Gold DNA polymerase and subjecting the reactions to 5 cycles of (94°C for 30 s, 45°C for 45 s and 72°C for 1 min).

Tagging step. The defined (+)TAG and (-)TAG sequences were incorporated onto the ends of the reporter sequences of each target RNA using primers **#3** and **#4** by addition of 75 µl of Tag Mix containing 0.2 µM each of primers **#3** and **#4** for each target RNA, 1X Taq buffer and 0.2 mM dNTPs, to the DNA reactions and subjecting the reactions to 5 cycles of (94°C for 30 s, 45°C for 45 s and 72°C for 1 min). Reactions were then

held at 72°C following the final cycle until retrieved from the thermocycler to inhibit production of primer dimers between the complex mixture of tagging primers, then placed on ice.

Multiplexed competitive amplification step. The resulting doublestranded DNA products from each of the target RNAs were then amplified with a common set of **TAG** primers by transferring 2 µl from the tagging reaction to 100 µl of MC reaction mix consisting of 1x Taq buffer, 0.2 mM dNTPs, 0.2 µM each of **TAG** primers and 5 U Amplitaq Gold, heating the reactions to 9.4°C for 10 min to activate the AmpliTaq Gold DNA polymerase and subjecting the reactions to 50 cycles of (94°C for 30 s, 60°C for 30 s and 72°C for 1 min). Aliquots (5 µl) from each reaction were analyzed on 4% agarose gels to insure that appropriate-sized product (180 bp) was produced.

Detection. The relative amounts of product produced from each of the target RNAs during the multiplexed competitive amplification step were quantified in the present studies by filter hybridization using probes specific for the reporter sequences amplified. For each target RNA analyzed in a given multiplex competitive reaction, 10 µl of product was combined with 1 ul of 2N NaOH/0.2 M EDTA in 1.5 ml Eppendorf centrifuge tubes and heated to 37°C for 30 min to denature the product (thus, if 3 targets were multiplexed, then 30 µl of product would be combined with 3 ml of sodium hydroxide solution). The tubes were then chilled on ice and 230 ml of ice cold 20x SSC was added per target RNA analyzed. For each target amplified, 200 ml of denatured product was immobilized onto nitrocellulose using a slot blotting apparatus

(Schleicher and Schuell). Replicate filters were prepared for hybridization to each reporter probe (oligonucleotides **#5** above) used for detection of the reporter sequences. Blots were hybridized overnight in Church buffer at 45°C, washed three times with 1x SSC at 45°C and radioactivity bound to the filters was quantified using a Molecular Dynamics phospholmager.

Data analysis. Changes in the level of a target RNA in an experimental RNA sample were compared to levels in a control RNA sample using the following formula, where **R**=hybridization signal generated from the experimental target RNA, **H**n=hybridization signals generated from house-keeping control RNAS, e = experimental RNA sample, c = control RNA sample:

% level of target RNA in experimental RNA sample relative to level in control RNA sample =

$100\% * \mathbf{R}e/\mathbf{R}c * (\mathbf{H}_1c+\mathbf{H}_2c+.....\mathbf{H}_nc)/(\mathbf{H}_1e+\mathbf{H}_2e+.....\mathbf{H}_ne)$

).

Referring to Figure 2, it compares Northern, RPA and MC-PCR analyses of a time course of stromelysin mRNA induction following IL-1 treatment. Total RNA was harvested from the HS27 cells at different time points following IL-1 addition to the growth medium. Stromelysin mRNA was analyzed either by Northern analysis using 5 µg total RNA (Figure 2A), RPA analysis of 2 µg total RNA (Figure 2B), or MC-PCR analysis of 1 µg total RNA (Figure 1C). GAPDH mRNA was used as the house-keeping control gene in the Northern and RPA assays to normalize for loading differences and for RNA integrity. GAPDH and β2-microglobulin were used as controls in the MC-PCR assays. In Figure 2D, stromelysin mRNA levels were calculated in Northern and

RPA studies by normalizing the hybridization signals of a probe to stromelysin RNA relative to hybridization signals of a probe to GAPDH RNA followed by the plotting of the normalized signals relative to levels observed in RNA from untreated HS27 cells. In the case of MC-PCR data, relative induction of stromelysin mRNA was calculated using the above formula. There was good agreement between the Northern and RPA assays with results from the MC-PCR assay, thus illustrating the quantitative nature of the MC-PCR assay.

Example 2: Sensitivity of MC-PCR

IL-1 induction of stromelysin mRNA in HS27 cells was used to address the sensitivity of the MC-PCR technique. Total RNA samples extracted from uninduced and IL-1-stimulated HS27 cells (17 hours post-induction) and 5 µg of RNA was analyzed by Northern analysis (Figure 3A) and 1 µg by MC-PCR (Figure 3B). In this experiment, β2-microglobulin was used as the control probe in the Northern analysis (Figure 3A). The Northern analysis indicated that stromelysin mRNA was induced about 30-fold; however, the stromelysin signal was difficult to quantify due to the low signal over background (see Figure 3A lane "Uninduced") which greatly affects the accuracy of measuring the magnitude of stromelysin mRNA induction. This problem was not encountered in the MC-PCR assay due to the inclusion of the PCR amplification step which amplified the stromelysin signal in the uninduced RNA sample well above noise (see Figure. 3B). The MC-PCR analysis indicated a 21-fold induction of stromelysin mRNA in the induced relative to the uninduced sample.

These same HS27 RNA samples were used to test the sensitivity of the MC-PCR assay. Varying amounts of RNA, ranging from 1 mg down to 0.01 µg, from either uninduced or IL-1 -stimulated HS27 cells was analyzed in triplicate as in Figure 3B and the hybridization signals were quantified and graphed (Figure 3C). Similar ratios of GAPDH, β2-microglobulin and stromelysin amplification products were obtained from either the untreated or IL-1 stimulated samples regardless of the amount of total RNA analyzed. Indeed, as little as 10 ng of total RNA, equivalent to the amount of RNA obtained from 1000 cells, was sufficient to obtain very reproducible results with MC-PCR (error bars represent standard deviations). The amount of RNA required for Northern analysis is 5 µg which is 500 times greater than the lowest amount of RNA tested in Figure 3C. These results demonstrate the superior sensitivity of the MC-PCR assay, compared to other techniques.

Example 3: Precision of MC-PCR reaction

The MC-PCR assay was sufficiently robust to detect differences in the induction levels of stromelysin mRNA in the above examples, which ranged from about 7-fold (Figure 2) to 21-fold (Figure 3). The ability of the MC-PCR assay to be used to discriminate smaller changes in stromelysin RNA was tested by diluting total RNA extracted from the IL-1 -stimulated HS27 cells used in Figure 3 with RNA from the untreated sample and measuring changes in stromelysin mRNA in the resulting admixtures (Figure 4). Changes in stromelysin mRNA levels were readily detected in all of the admixtures

indicating the ability of the assay to identify small differences (less than 2-fold) in mRNA abundances.

Example 4: MC-PCR analysis of low-copy number mRNAs.

GAPDH, β2-microglobulin and stromelysin mRNAs represent relatively abundant mRNAs (greater than 1000 copies per cell) in IL-1 -stimulated HS27 cells. To test whether changes in low abundance mRNAs could also be analyzed with the MC-PCR method, we compared results obtained from Northern and MC-PCR analyses of serum induction of *c-fos* mRNA in HeLa cells. *c-fos* mRNA is extremely low in serum-starved HeLa cells, but is induced more than 20-fold 30 minutes after addition of serum (Figure 5A). Despite this large induction of Fos mRNA, 30 µg of total RNA was required to detect Fos RNA by Northern analysis even after long exposure of the autoradiograph (Figure 5A). The same uninduced and 30-minute time point RNA samples then analyzed by MC-PCR (Figure 5B). Because of the greater sensitivity of the MC-PCR assay (Figure 4), only 1 µg of RNA was required to quantify Fos mRNA induction in the sample from the 30-minute time-point (Figure 5B). However, because of the abundance of GAPDH and β2-microglobulin mRNAs, they were not useful as controls for MC-PCR analyses of *c-fos* mRNA induction. Consequently, other less abundant mRNAs were used as controls to quantify changes in *c-fos* mRNA levels by MC-PCR. cdc2 kinase and c-raf mRNAs were used as controls in these experiments because they are less abundant than either GAP or β2-microglobulin mRNAs. The magnitude of *c-fos* mRNA induction in the RNA was the same (about 30-fold) as determined by either Northern or MC-PCR assays. These results again demonstrate that the

MC-PCR assay is comparable to Northern analysis for measuring changes in low-abundance mRNAs, but overall the MC-PCR is superior because of its greater sensitivity.

Example 5: MC-PCR analysis of antisense inhibition of cdc2 kinase mRNA

Referring to Figure 6, HeLa cells were treated for 4 hours with either a phosphorothioate antisense oligonucleotide targeting cdc2 kinase mRNA or a mismatch control, complexed at a 4:1 charge ratio with Pfx4 (Life Technologies) in OptiMEM® (BRL/Gibco). Cells were then washed, cultured for an additional 20 hours in complete media, RNA was harvested and 400 ng was analyzed by MC-PCR using oligonucleotides specific for cdc2 kinase, and K-ras (ras) as a control target RNA. Error bars represent standard deviations. As shown in the figure 6, cells treated with antisense oligonucleotide targeted against cdc2 RNA showed a 50% reduction in the level of cdc2 RNA compared to cells treated with a mismatched control oligonucleotide. This shows that the MC-PCR technique can detect changes in the relative abundance of a target RNA in response to specific inhibition.

Uses

The present invention can be used to quantify changes in abundance of target DNA or RNA sequences in response to any external or environmental stimuli including but not limited to antisense, ribozymes or drug treatment can be readily accomplished using the instant invention.

Quantifying changes in abundance of target DNA or RNA sequences during development or differentiation of a cell type or tissue, or during development or abbrogation of a particular disease phenotype, can be readily accomplished using the instant invention.

The instant invention can be used to detect the presence of, and/or quantify the relative amounts of, infectious agents including but not limited to viruses, bacteria, fungi, protazoa or the like in biological samples for diagnostic or prognostic purposes.

Detecting or quantifying presence of exogenous DNA or RNA sequences including but not limited to vectors or the like used for the purposes of gene therapy.

For use in screens to discover or evaluate agents that either inhibit or promote expression of a gene(s) of interest.

Other embodiments are within the following claims.

CLAIMS

1. A process for measuring the relative amounts of two or more different nucleic acid molecules in a system, comprising the steps of:

a) converting said nucleic acid molecules to tagged molecules using a 5'- and a 3'- primer specific for each said nucleic acid molecule, said 5' primers each comprising a first defined sequence and said 3' primers each comprising a second defined sequence;

b) amplifying said tagged molecules together in one reaction vessel with a generic primer pair, wherein one said generic primer comprises a sequence complementary to said first defined sequence, the other said generic primer comprises a sequence complementary to said second defined sequence, to produce amplified tagged nucleic acid molecules; and

c) measuring the relative amount of each said amplified tagged molecule as a measure of the relative amount of said different nucleic acid molecules.

2. The process of claim 1, wherein said nucleic acid molecules are converted into tagged DNA molecules.

3. The process of claim 2, wherein the relative amount of at least two different nucleic acid molecules is measured, wherein one said nucleic acid is encoded by a target gene, and the other said nucleic acid is encoded by one or more control genes.

4. The process of claim 3, wherein said control gene is a house-keeping gene.

5. The process of claim 4, wherein said house-keeping gene is selected from a group consisting of β-actin, β2-microglobulin and glyceraldehyde-3-phosphate dehydrogenase.

6. The process of claim 3, wherein said target gene is derived from an infectious agent selected from a group consisting of viruses, bacteria, fungi and protozoa.

7. The process of claim 3, wherein said target gene is encoded by a plant genome.

8. The process of claims 1 or 3, wherein said nucleic acid molecule is RNA.

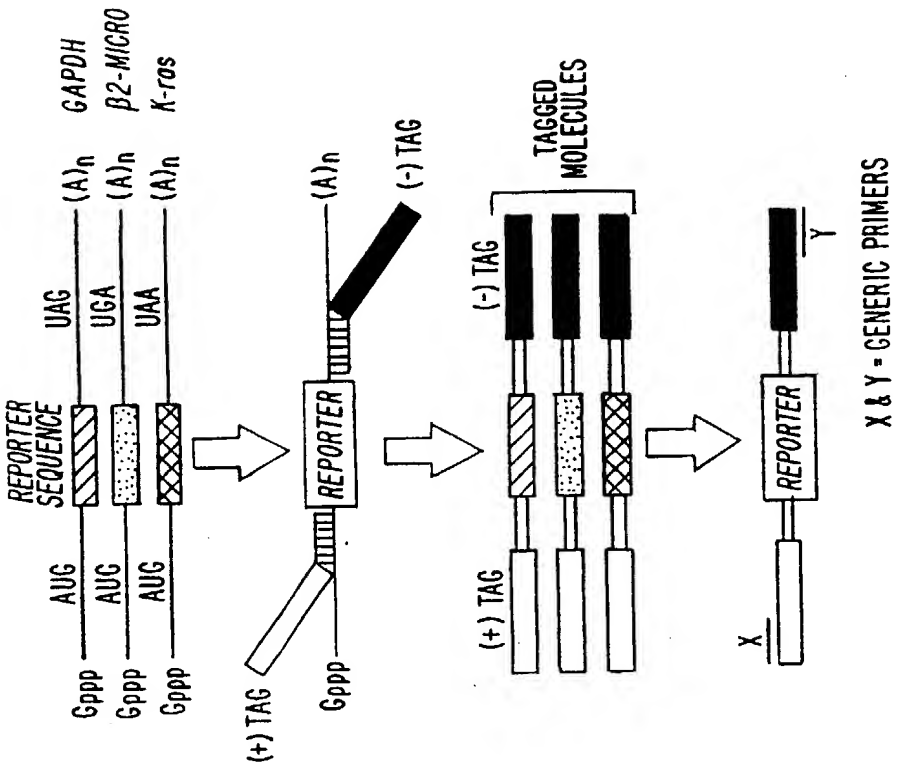
9. The process of claims 1 or 3, wherein said nucleic acid molecule is DNA.

10. The process for claim 8 comprising the steps of:
a) converting said RNA molecules to single stranded complementary DNA molecules;
b) converting the single stranded complementary DNA molecules into double stranded DNA molecules;
c) converting the double stranded DNA molecules into tagged molecules using a 5'- and a 3'- primer specific for each said double stranded DNA molecule, said 5' primers each comprising a first defined sequence and said 3' primers each comprising a second defined sequence;

d) amplifying said tagged molecules together in one reaction vessel with a generic primer pair, wherein one said generic primer comprises a sequence complementary to said first defined sequence, the other said generic primer comprises a sequence complementary to said second defined sequence, to produce amplified tagged nucleic acid molecules; and

e) measuring the relative amount of each said amplified tagged molecule as a measure of the relative amount of said different RNA molecules.

FIG. 1A.



- RNAs
  - -PATTERNS INDICATE RNA-SPECIFIC REPORTER SEQUENCES TO BE AMPLIFIED
- RT AND TAGGING REACTION
  - -cDNA SYNTHESIS
  - -SPECIFIC PRIMERS ARE USED TO ADD DEFINED PRIMER SEQUENCES 5' AND 3' TO THE REPORTER SEQUENCES
- COMPETITIVE AMPLIFICATION
  - -GENERIC PRIMER PAIR USED TO AMPLIFY THE VARIOUS TAGGED MOLECULES
  - -AMPLIFICATION REACTION IS CARRIED OUT TO EQUILIBRIUM

FIG. 1B.

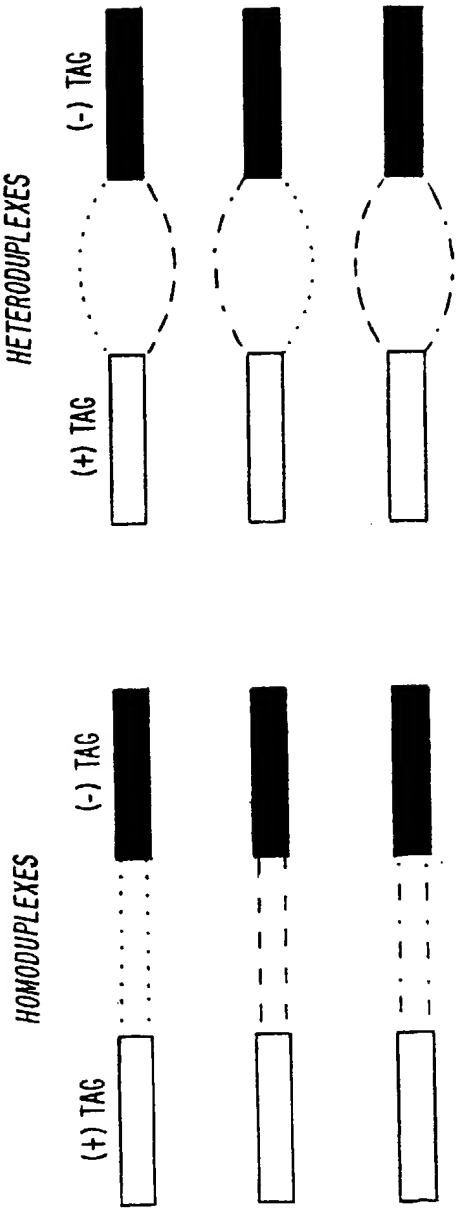
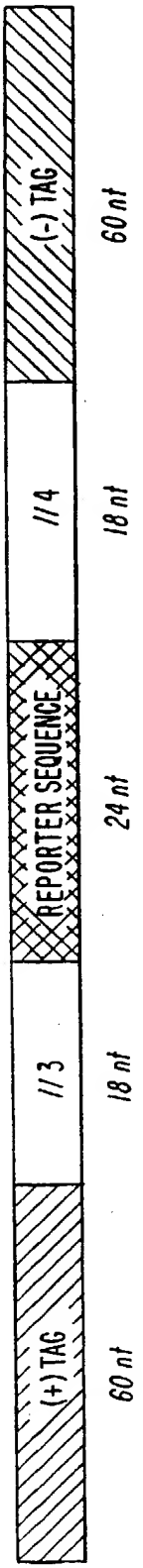


FIG. 1C.

AN EXAMPLE OF A MULTIPLEX COMPETITIVE PCR PRODUCT



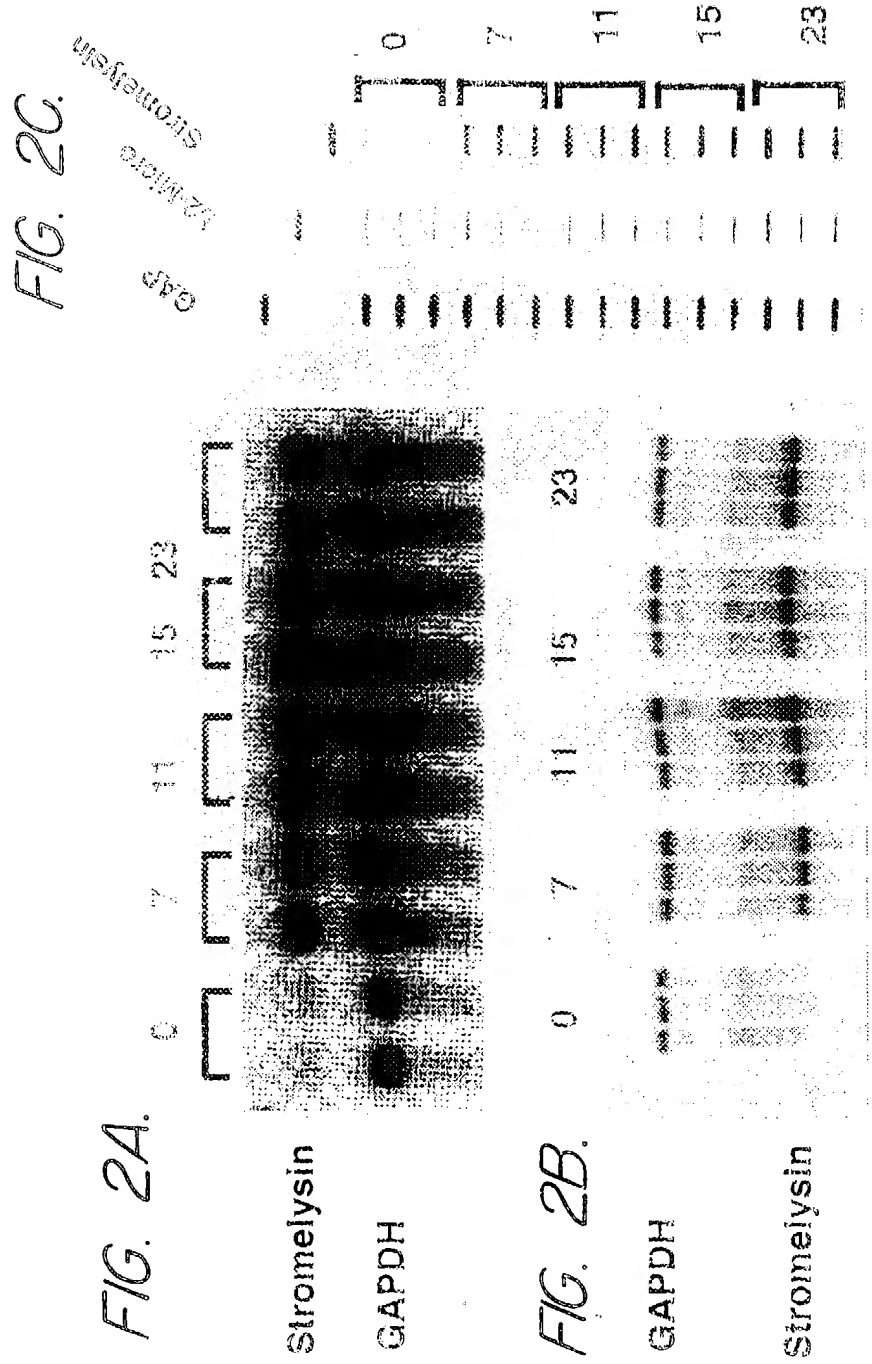
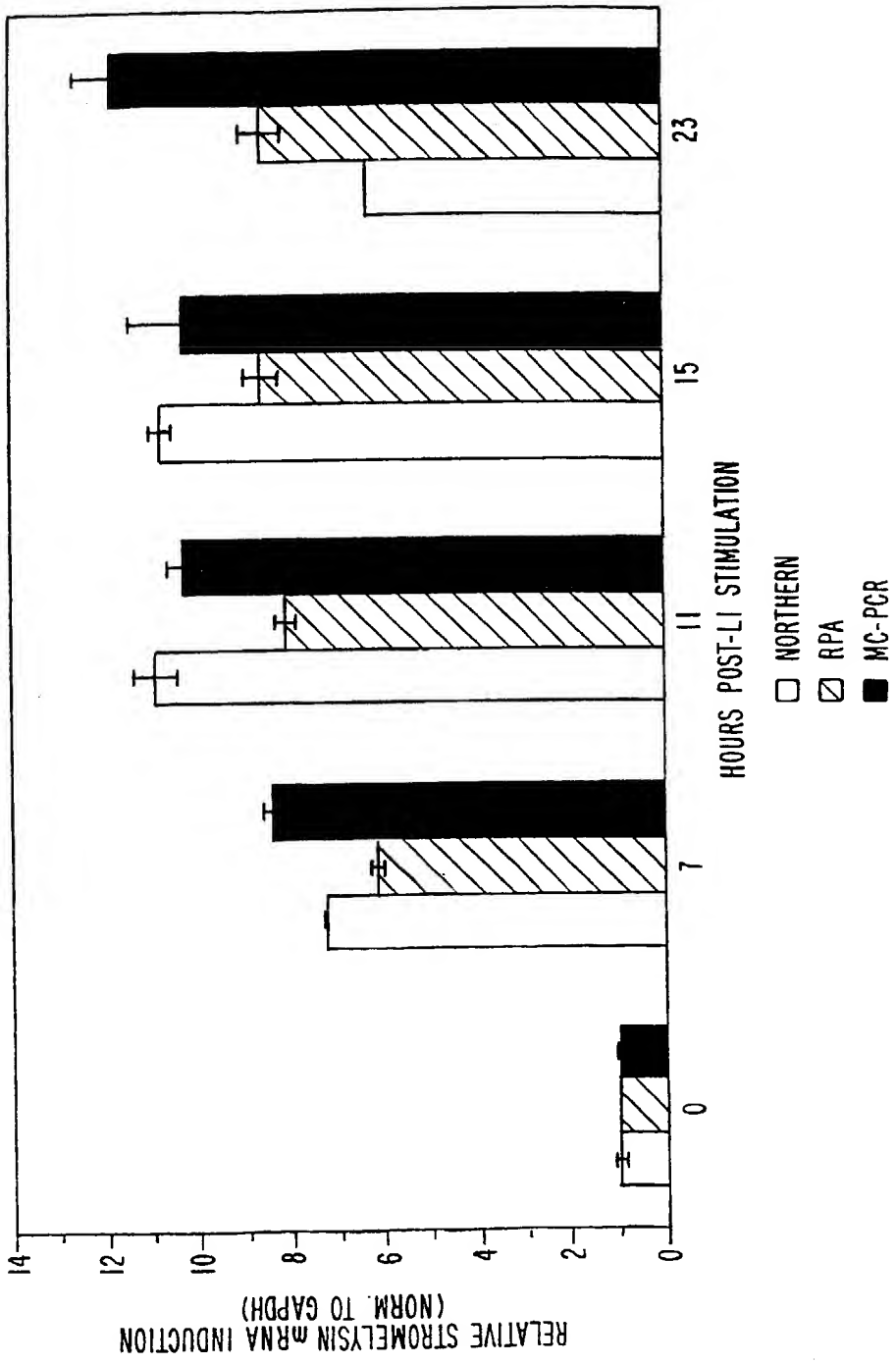


FIG. 2D.



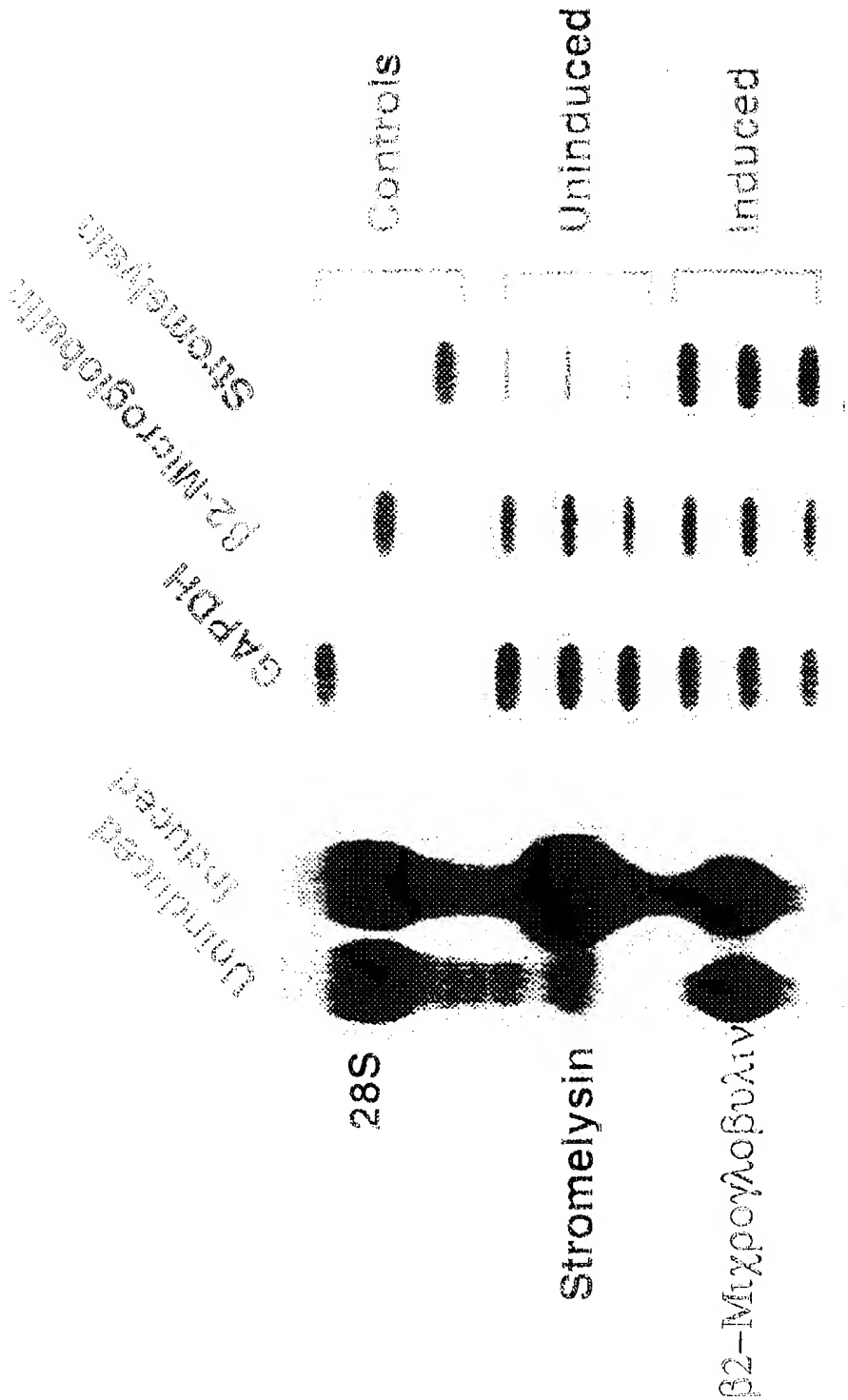


FIG. 3B.

FIG. 3A.

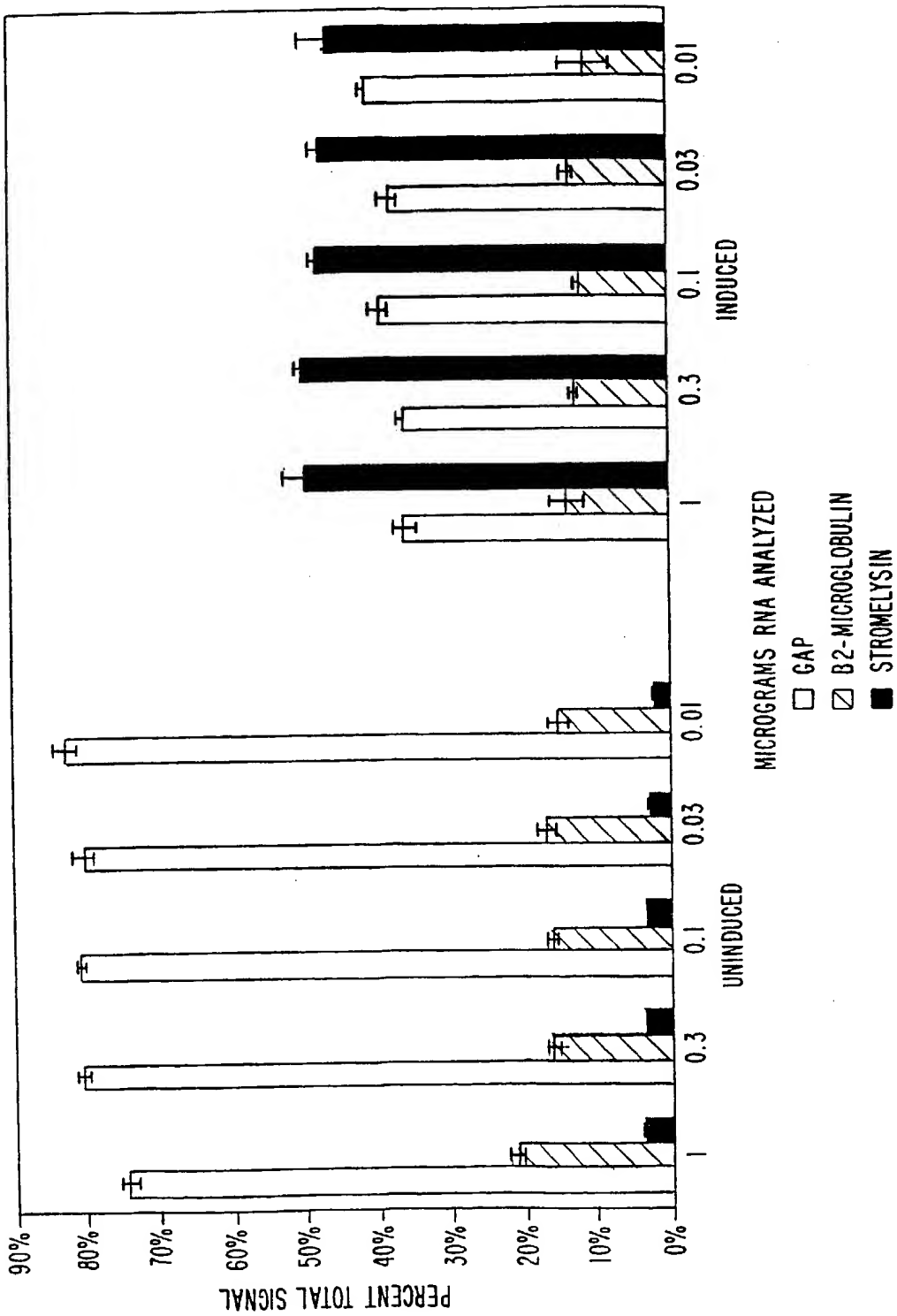


FIG. 3C.

FIG. 4.

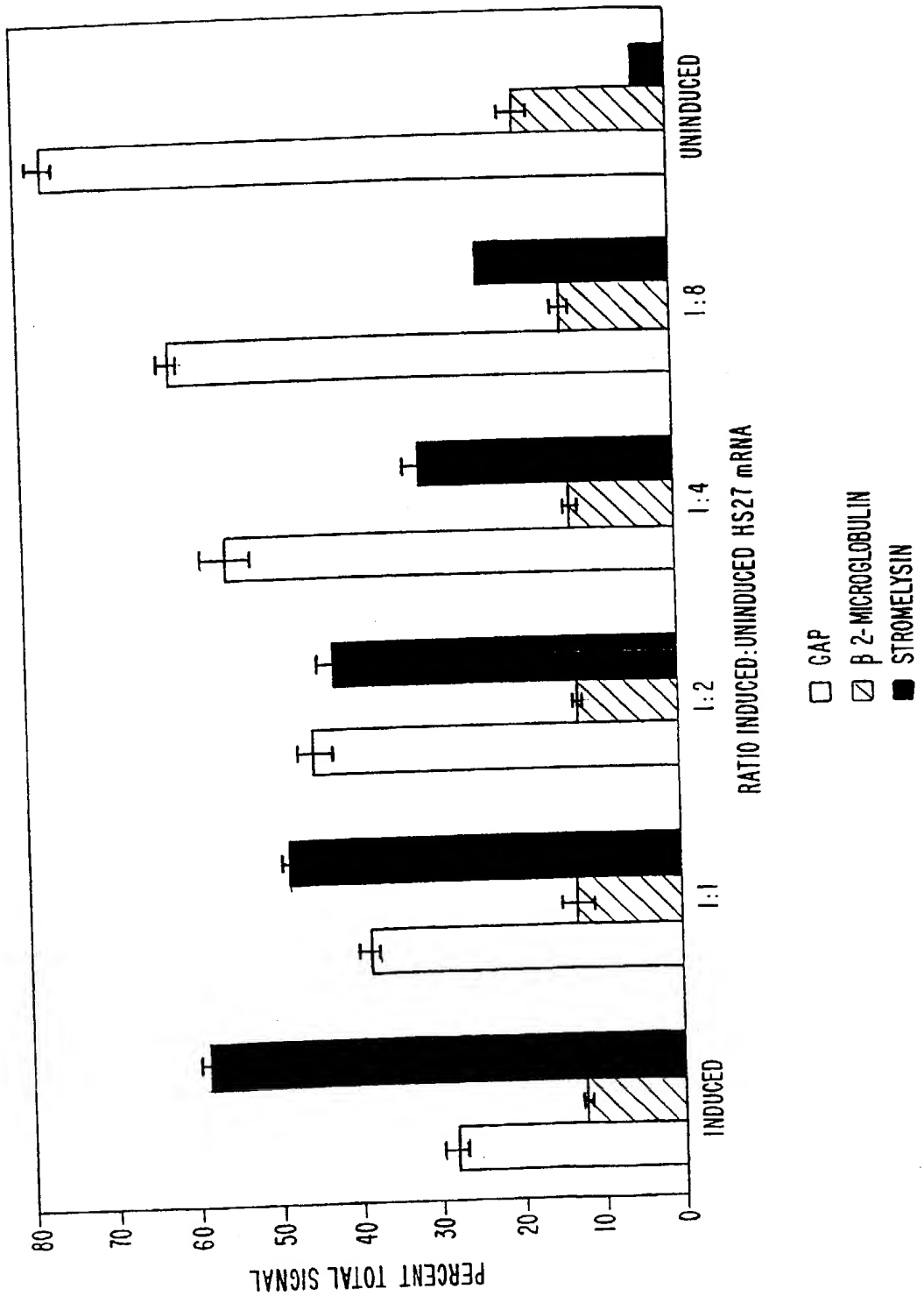


FIG. 5A.

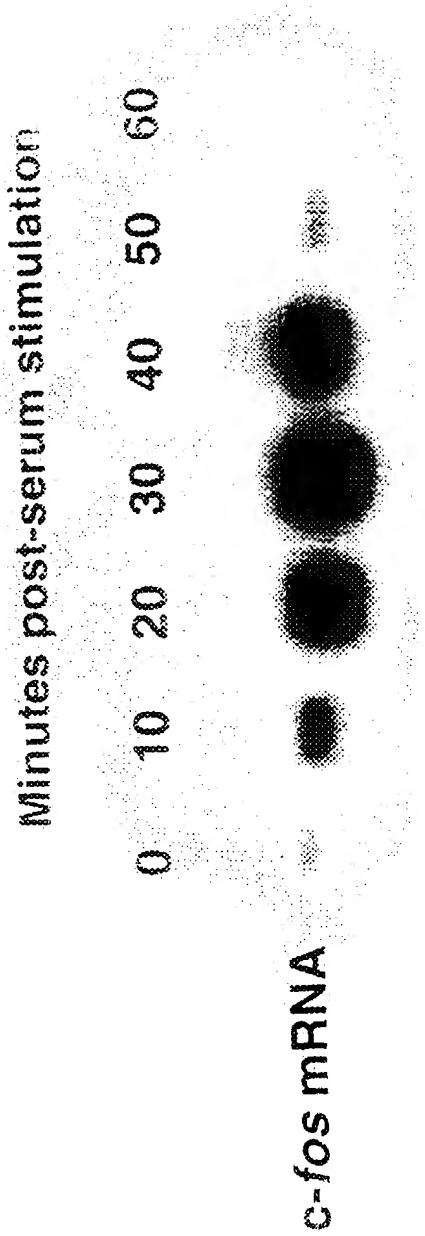


FIG. 5B.

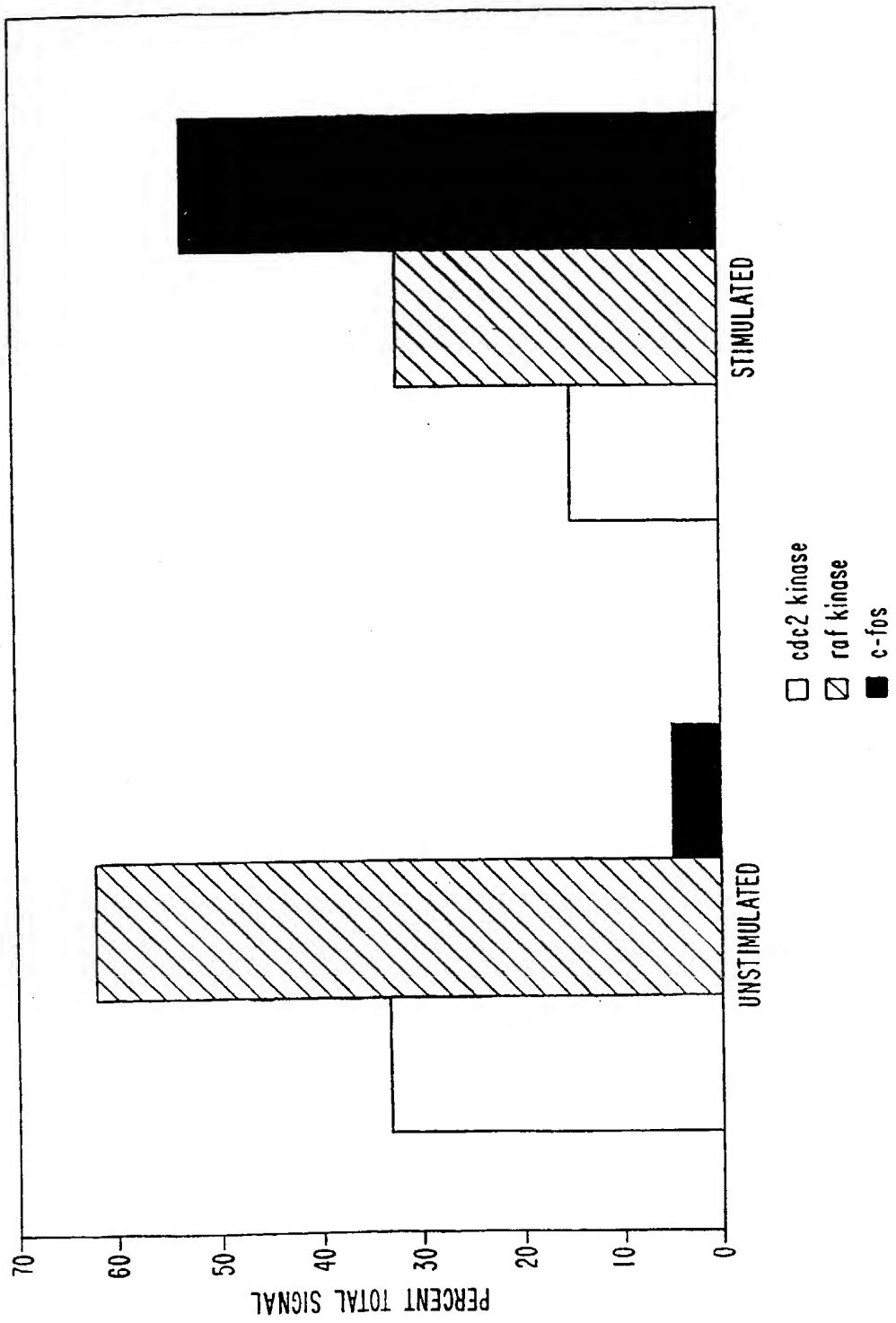
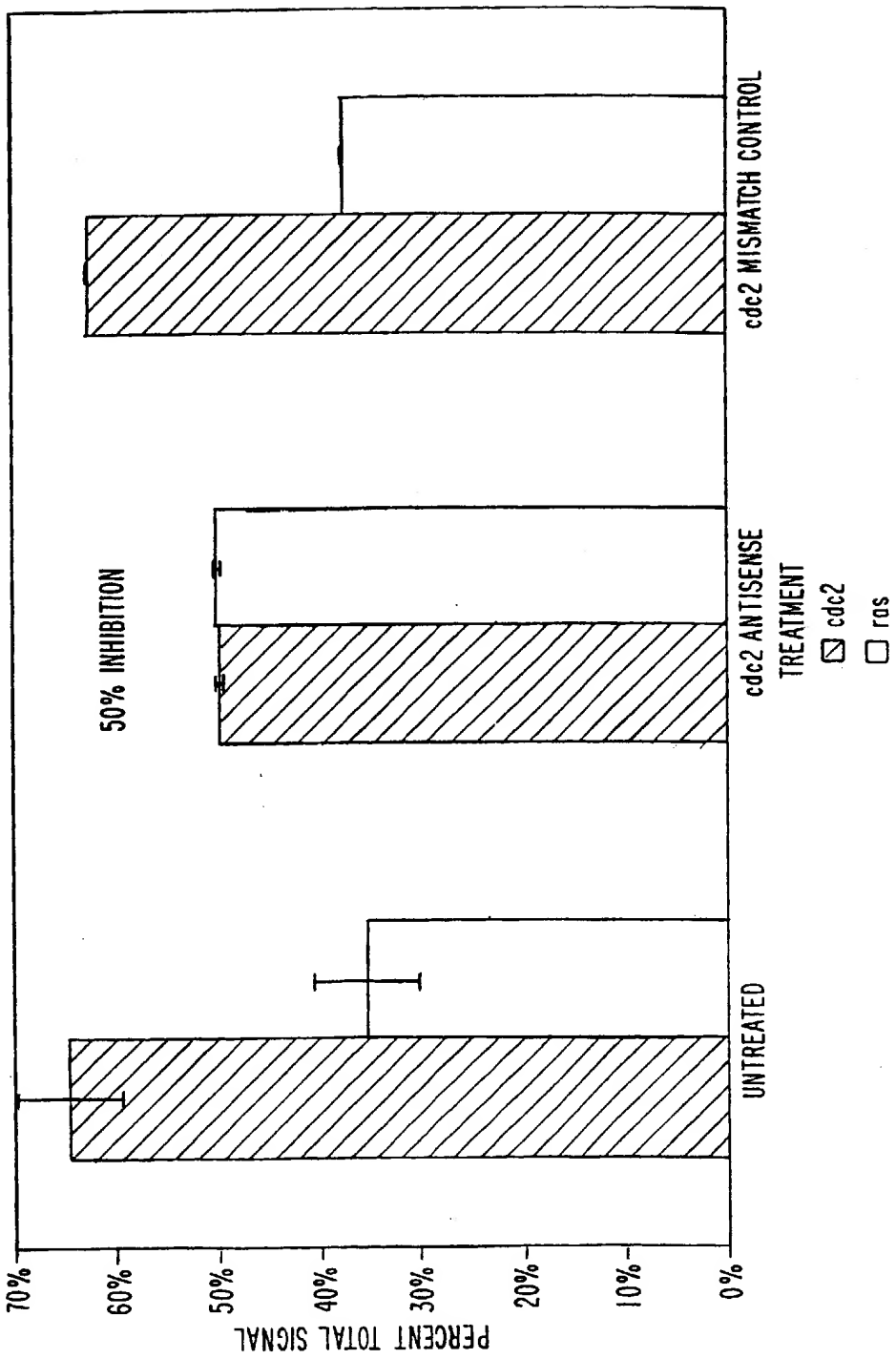


FIG. 6.



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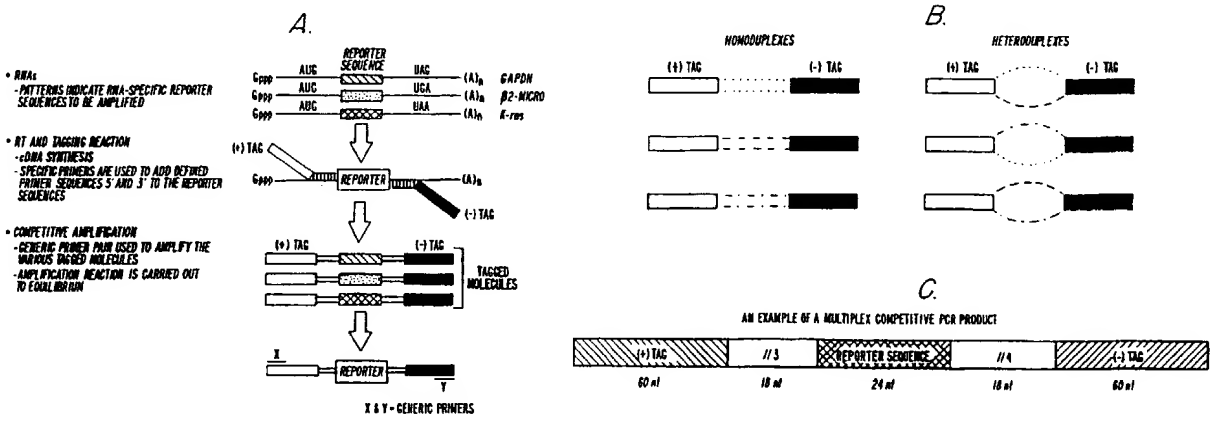

(57) Abstract

A process for measuring the relative amounts of two or more different nucleic acid molecules in a biological system using a multiplex competitive polymerase chain reaction.

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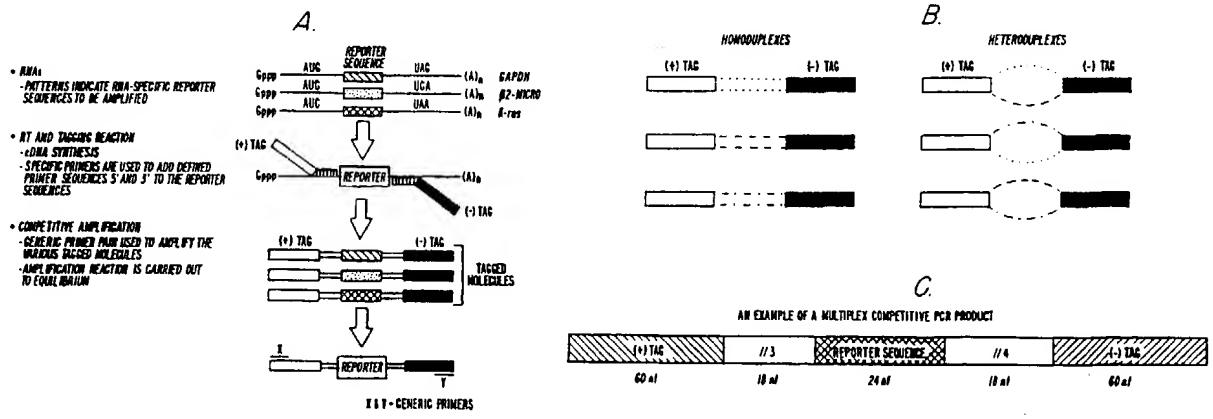
(54) Title: IMPROVED PROCESS FOR DETECTION AND QUANTIFICATION OF NUCLEIC ACID MOLECULES



(57) Abstract

A process for measuring the relative amounts of two or more different nucleic acid molecules in a biological system using a multiplex competitive polymerase chain reaction.

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